AD_____

Award Number: W81XWH-04-1-0438

TITLE: Met Nuclear Localization and Signaling in Breast Cancer

PRINCIPAL INVESTIGATOR: Sharon A. Pozner Moulis

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, Connecticut 06520-8047

REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual Summary (2 Apr 2004 - 1 Apr 2005)

4. TITLE AND SUBTITLE
Met Nuclear Localization and Signaling in Breast Cancer

5. FUNDING NUMBERS
W81XWH-04-1-0438

6. AUTHOR(S)
Sharon A. Pozner Moulis

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Yale University School of Medicine
New Haven, Connecticut 06520-8047

E-Mail: Sharon.pozner@yale.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Hepatocyte Growth Factor/Met signaling is associated with tumor aggression and poor prognosis in many cancers, including lymph node negative breast carcinomas. High HGF levels have been correlated with worse patient outcome and over-expression of the cytoplasmic region of Met is associated with poor prognosis in lymph node negative breast carcinomas. Immunohistologic assay, using antibodies to the cytoplasmic domain of Met, show Met in the nucleus in cell lines and in germinal regions of tissues. Cell fractionation of A431 and HEK293 cells reveal a 60kDa band recognized by C-terminal antibodies of Met localizing to the nucleus. This 60kDa fragment can be enriched by the presence of proteosome inhibitors and is independent of HGF treatment. GFP fusion proteins of the cytoplasmic domain of Met transfected into HEK293 cells are found in the nucleus while the full length Met-GFP fusion is membranous. Further deletions of the Met-GFP fusions identify a region of the juxtamembrane domain required for nuclear translocation. This work suggests processing of the Met receptor, in a manner similar to ErbB4, resulting in the release of the cytoplasmic domain and its translocation to the nucleus.

14. SUBJECT TERMS	15. NUMBER OF PAGES
c-met, receptor tyrosine kinase, cleavage	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:
The Hepatocyte Growth Factor/Met signaling pathway plays an important role in embryonic development, mammary gland formation and the epithelial-mesenchymal transition. Overexpression of Met is associated with tumor aggression and poor prognosis in many cancers including lymph node negative breast carcinoma. A previous study showed that it is the Met cytoplasmic domain, and not the extracellular domain that is correlated with poor patient outcome in lymph node negative breast carcinomas [1]. This difference is most likely explained by a processing event. Other receptor tyrosine kinases have been shown to be cleaved and the resultant cleavage product translocates to the nucleus as in the case of ErbB4 [2]. Although Met has not previously been reported to undergo a similar cleavage, here I show that a 60kDa fragment of Met localizes to the nucleus in a ligand-independent manner. The purpose of this study is to verify the presence of the cytoplasmic domain of Met in the nucleus and determine the mechanism by which nuclear translocation occurs.

Body:
The goal of this study is to verify the presence of the Met receptor in the nucleus of cell lines. Following confirmation of this phenomenon, the goal becomes to determine the mechanism by which Met translocates to the nucleus and the role this plays in HGF-Met signaling.

The original statement of work was as follows:

Task 1. To confirm nuclear localization of the cytoplasmic domain of the Met receptor in HMEC cells, Months 0-24:
- a. Construction and transfection of GFP-tagged Met cytoplasmic domain, Months 0-3
- b. Subcellular fractionation, Months 3-6
- c. Identification of Met antibodies for use on tissue microarray of normal and cancerous cells, Months 12-24

Task 2. Definition of the domain responsible for translocation to the nucleus, Months 6-30):
- a. Construct and transfection of truncated versions of GFP-tagged cytoplasmic Met, Months 6-12
- b. Subcellular fractionation, Months 12-18
- c. Characterization of turnover rate, 18-30

Task 3. Definition of the cleavage domain and cleavage site, Months 18-36:
- a. Tag full length Met and transfect into cells to verify cleavage, Months 18-21
- b. Bioinformatics to determine and test protease candidates, Months 21-27
- c. Create mutant Met unable to be cleaved, Months 27-30
- d. Characterize mutant and wild type effect on cells using growth assays, Months 30–36+

Task 1 was successfully completed during this first year of the grant. Verification of a 60kDa C-terminal fragment of Met localizing to the nucleus was confirmed through western blotting,

immunoprecipitation, northern blotting, cellular fractionation and construction and transfection of a GFP-tagged construct of the Met cytoplasmic domain.

Immunohistochemistry, using an antibody to the C-terminus of the Met receptor, shows Met in the cytoplasm and nucleus of normal and cancerous tissues rather than the membrane as one would expect (Figure 1a). Normal colon, skin and testis show nuclear staining of Met in germinal tissue layers and aberrant expression throughout cancerous tissue. The transition to Met overexpressing cancers is most dramatically seen in lymphoma tissue where Met is completely absent in the normal lymph node tissue and localized to the nucleus in cancerous tissue. Do to variability of the Met antibodies these results were further confirmed using a four different antibodies to the cytoplasmic domain of Met. These antibodies were C12, C28 and CVD13 polyclonal antibodies to the C-terminus of Met and 3D4, a monoclonal antibody to the tyrosine kinase domain. Mammary epithelial lines HMEC and MCF10-A, melanoma cell lines Mel1241 and Mel1335 (data not shown), epidermoid carcinoma line A431, and colon carcinoma line HT29 all showed nuclear staining either in all cells, or in cells along the periphery of growing colonies (Figure 1b).

To verify the presence of Met in the nucleus of cell lines, cellular fractionation of high and low Met expressing cells was carried out. Separation of the cytoplasm from the nucleus resulted in a 60kDa fragment, recognized by antibodies to the C-terminus of Met, localizing to the nuclear fraction, while the 145kDa Met receptor remained in the cytoplasmic fraction in both A431 (high expressor) and HEK293 (low expressor) cell lines (Figure 2). Additional bands in the cytoplasmic fraction of A431 cell lysate are most likely due to breakdown of the protein. These results confirm the presence of a 60kDa fragment of Met in the nucleus. However, do to the unreliability or possible cross-reactivity of some of these antibodies, more rigorous tests were done to confirm that the fragment detected is Met. Nuclear staining of Met was seen in cell lines previously considered low expressors or negative for Met, therefore a northern blot was done to confirm the presence of Met in these cell lines. Using a radioactively-labeled probe made from the cytoplasmic domain of Met, a 9.5kb transcript was detected in all cell lines. The smaller 7.5kb variant of Met was also seen in all cell lines except NIH3T3 and Mel1335 (Figure 3). From this, it was concluded that cell lines showing nuclear localization of Met do indeed express Met and the nuclear localization of the protein is not derived from a novel alternative splicing variant.

To further confirm that the 60 kDa fragment was Met a series of western blots and immunoprecipitations were carried out. Cell lysate from A431 cells show the presence of Met on a western blot. A431 cells were serum starved and treated with ALLN, a proteasome inhibitor. Treatment with ALLN resulted in the appearance of a band of approximately 60kDa in size detected by three different antibodies to Met (Figure 4a). Untreated HEK293 cells also showed the presence of a 60kDa band (Figure 5), suggesting the presence of this fragment does not need to be stabilized by a proteasome inhibitor in some cell lines. Immunoprecipitation of Met from ALLN treated and untreated A431 cells as well as untreated HEK293 cells and NIH3T3 cells show a band of 60kDa (Figure 4b). These results show a basal level of a 60kDa fragment of Met present in cell lines with varying expression levels of Met. Expression of the 60kDa fragment can be increased with treatment of a proteasome inhibitor, ALLN, suggesting that the 60kDa fragment may be the product of a processing event of the Met receptor.

Surprisingly, treatment with HGF did not induce the appearance of this fragment, nor did simultaneous treatment with ALLN and HGF affect the presence of the 60 kDa fragment (Figure 4c).

The cytoplasmic domain of Met was ligated into a GFP expressing vector such that GFP would be located on the C-terminus of the Met construct. The construct was transiently transfected into HEK293 cells and the presence of the Met construct was confirmed by western blot (Figure 5b). The cytoplasmic Met construct localizes to the nucleus of these cells whereas the full-length Met construct is restricted to the cell membrane (Figure 5c).

Task 2 was to define and the domain responsible for nuclear translocation and to characterize the cleavage event. This was partially completed during the first year of this grant.
To determine the domain responsible for nuclear translocation of Met a series of N-terminally truncated constructs were created with a GFP tag on the C-terminus as seen in figure 5a. These include a the full length Met protein, the cytoplasmic domain truncated at the transmembrane/intracellular junction (K956), three constructs truncated after tyrosine residues in the juxtamembrane region (D972, R1004, P1027), the tyrosine kinase domain (I1084) and a truncated tyrosine kinase domain (L1157). As described above, the cytoplasmic domain construct localizes to the nucleus. Constructs lacking the juxtamembrane region P1027-I1084 do not show nuclear enrichment. This region of the juxtamembrane domain does not appear to have any known nuclear localization sequences but is required for nuclear localization of the cytoplasmic domain of Met.

Work on task 3 has begun with no results to report as of yet. Although there has been no progress on identifying a stimulant for Met cleavage, it has been observed that this phenomenon is cell density dependent. As seen in Figure 6, a confluent monolayer of A431 cells have Met in the nucleus whereas a superconfluent monolayer of cells show Met at the cell membrane as would be expected. This would suggest that nuclear Met plays a role in the epithelial to mesenchymal transition (as does the Met receptor). Further characterization of this phenomenon as well as attempts to isolate proteases responsible for Met cleavage are currently underway.

Key Research Accomplishment:

- Verification of Met in the Nucleus
- Identification of region responsible for Met nuclear localization

Reportable Outcomes:

Poster Presentation: San Antonio Breast Cancer Research Symposium, December 2004

Conclusions:

I have demonstrated for the first time that a C-terminal fragment of the HGF receptor Met localizes to the nucleus in a ligand-independent manner. Using a series of monoclonal and polyclonal antibodies to the C-terminus of Met, we show that Met expression is present not only at the membrane, but also in the cytoplasm and nucleus in 10 cell lines and predominantly in germinal regions in a range of tissues from normal organs. Cell lines expressing nuclear Met have a mesenchymal phenotype or are on the leading edge of epithelial clusters. All cell lines expressing Met in the nucleus show Met transcripts by Northern Blot. Western blotting reveals a 60kDa band recognized by antibodies to the C-terminus of Met that localizes to the nucleus. The appearance of a smaller protein recognized by Met antibodies in the nucleus and the lack of a smaller transcript by Northern Blot would imply that this fragment is derived from the full length Met receptor by a processing event. Although no nuclear localization sequence has been identified, serial deletion constructs of Met isolate a region of the juxtamembrane domain (P1027-I1084) that is required for nuclear localization of the cytoplasmic fragment of Met. This fragment is not an alternative splice as shown by Northern blotting and is most likely the product of a cleavage event as the full length Met-GFP fusion does not localize to the nucleus.

A previous study showed that it is the Met cytoplasmic domain, and not the extracellular domain that is correlated with poor patient outcome in lymph node negative breast carcinomas [1]. This difference is most likely explained by a processing event. Here, I show the first evidence that the Met receptor may undergo a processing event resulting in translocation to the nucleus. The presence of Met in the nucleus indicates that it may play a role in enhancing signaling of the full length Met receptor or may be indicative of a novel signaling pathway that correlates with poor patient outcome in lymph node negative breast carcinoma.

References:

1. Kang, J.Y., et al., *Tissue microarray analysis of hepatocyte growth factor/Met pathway components reveals a role for Met, matriptase, and hepatocyte growth factor activator inhibitor 1 in the progression of node-negative breast cancer.* Cancer Res, 2003. **63**(5): p. 1101-5.

2. Ni, C.Y., et al., *gamma -Secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase.* Science, 2001. **294**(5549): p. 2179-81.

Appendix 1: Figures

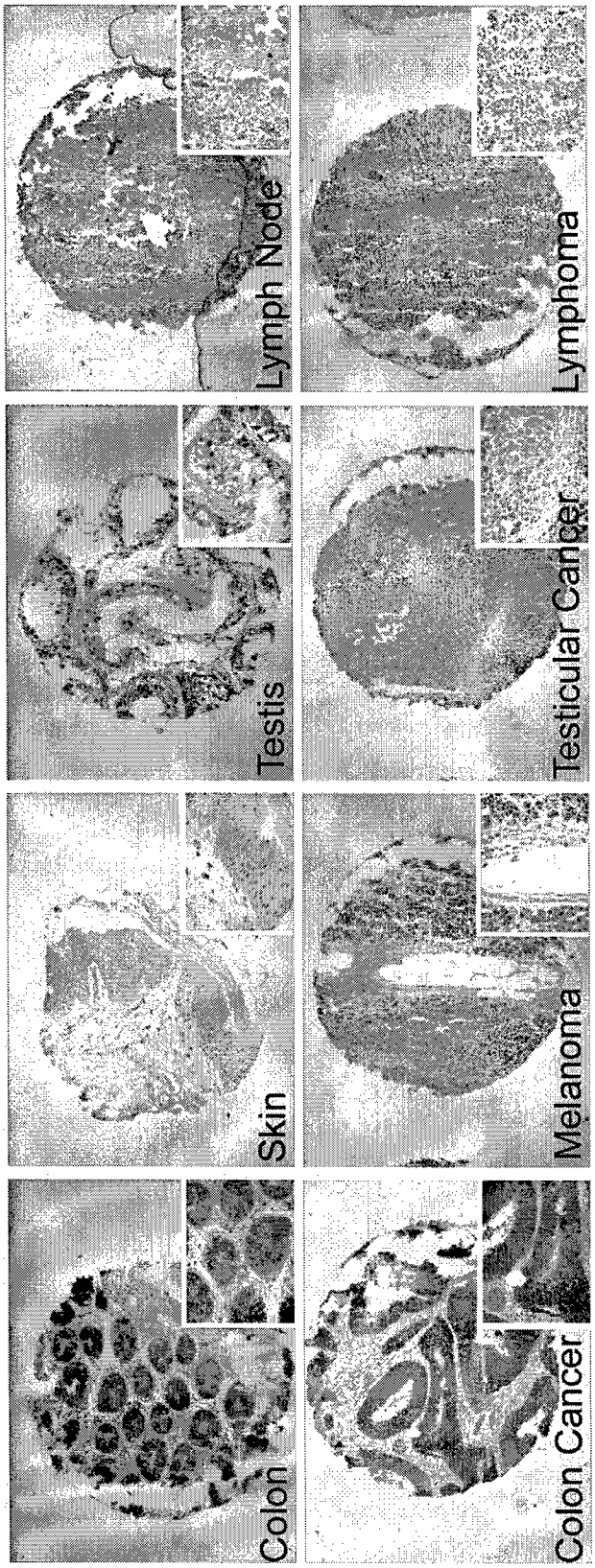

Figure 1a

Figure 1b

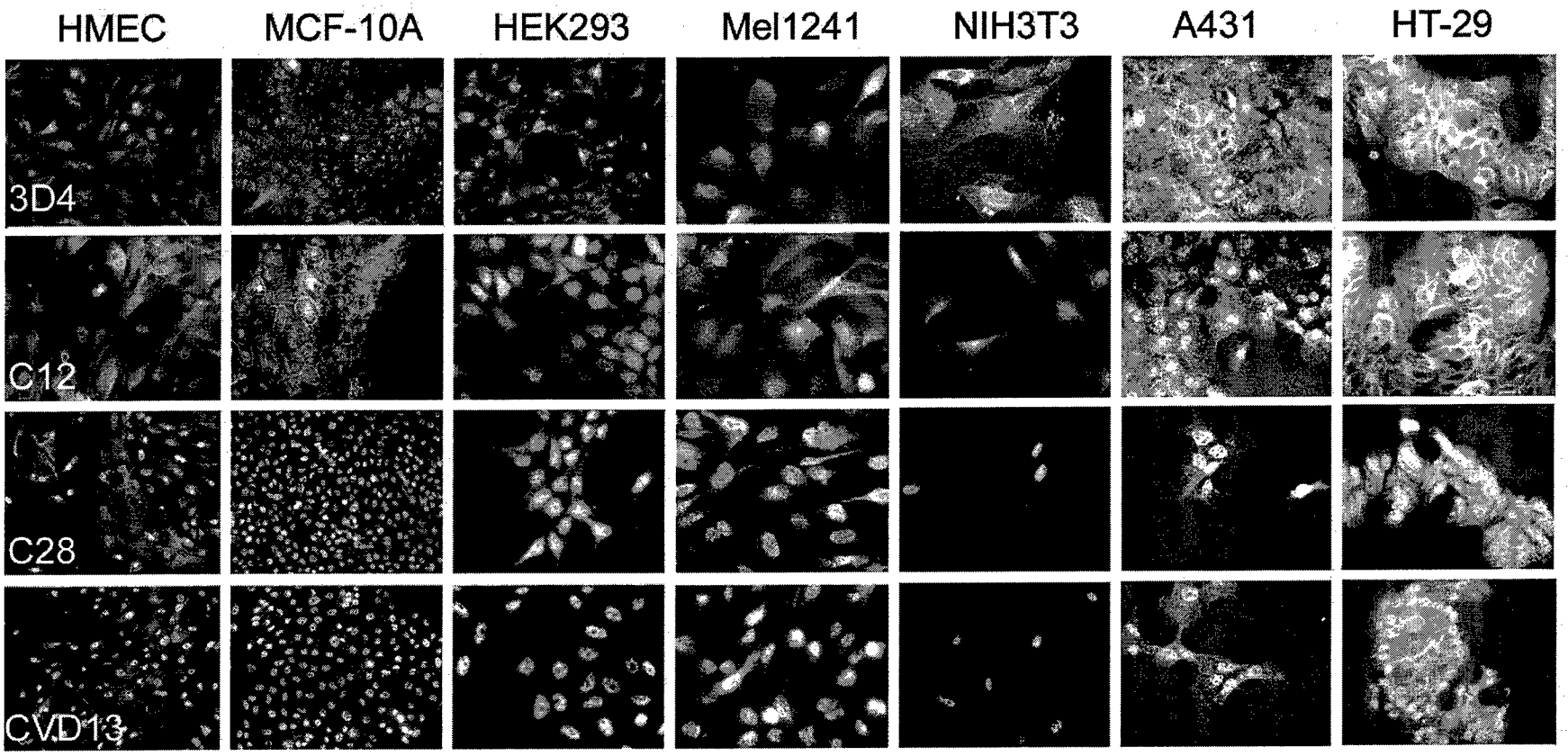
HMEC
MCF-10A
HEK293
Mel1241
NIH3T3
A431
HT-29
3D4
C12
C28
CVD13

Figure 2

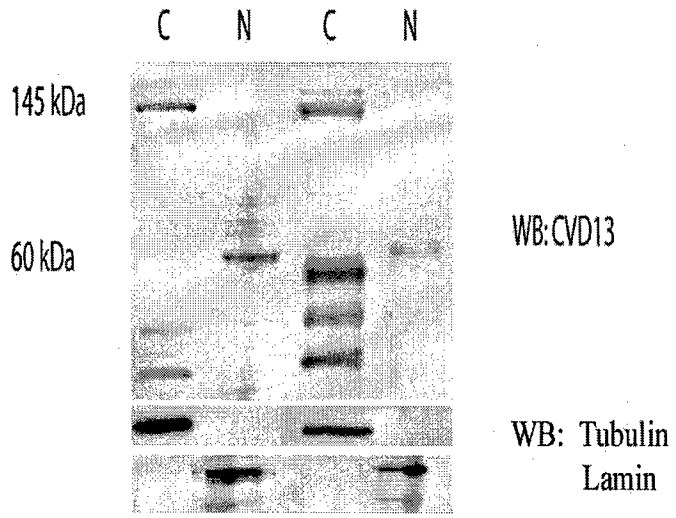

Figure 3

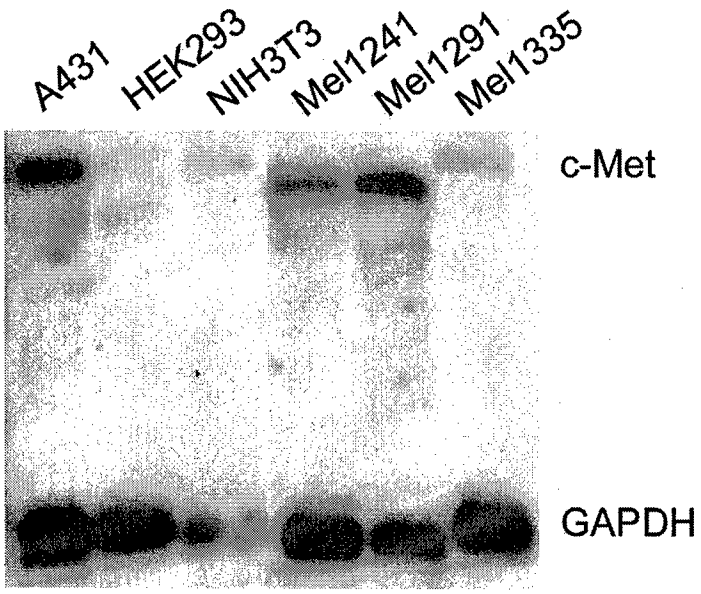

Figure 4

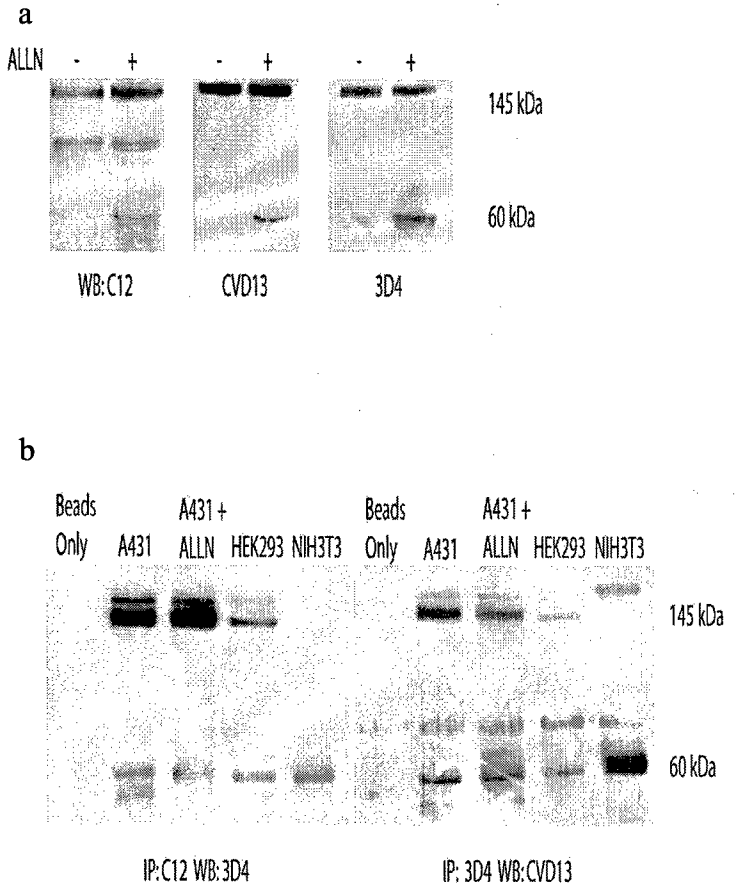
a
ALLN - + - + - +
145 kDa
60 kDa
WB:C12
CVD13
3D4
b
Beads Only
A431
A431 + ALLN
HEK293
NIH3T3
Beads Only
A431
A431 + ALLN
HEK293
NIH3T3
145 kDa
60 kDa
IP:C12 WB:3D4
IP: 3D4 WB:CVD13
c
10' 30' 60'
HGF - - + + + +
ALLN - + - + + +
-145 kD
-60 kD
WB: 3D4
IP: C12
WB:4G10

Figure 5

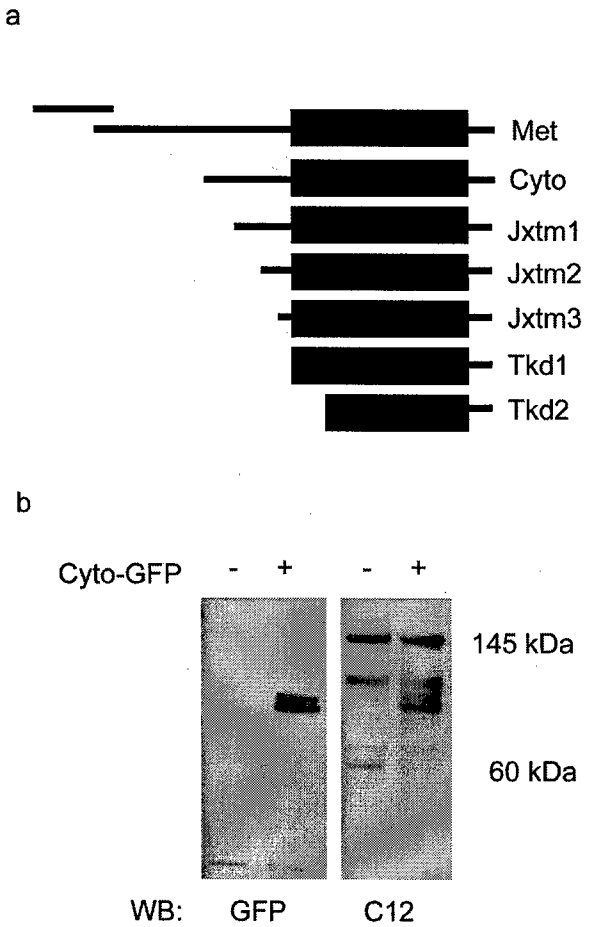
a
Met
Cyto
Jxtm1
Jxtm2
Jxtm3
Tkd1
Tkd2
b
Cyto-GFP - + - +
145 kDa
60 kDa
WB: GFP C12

Figure 5c

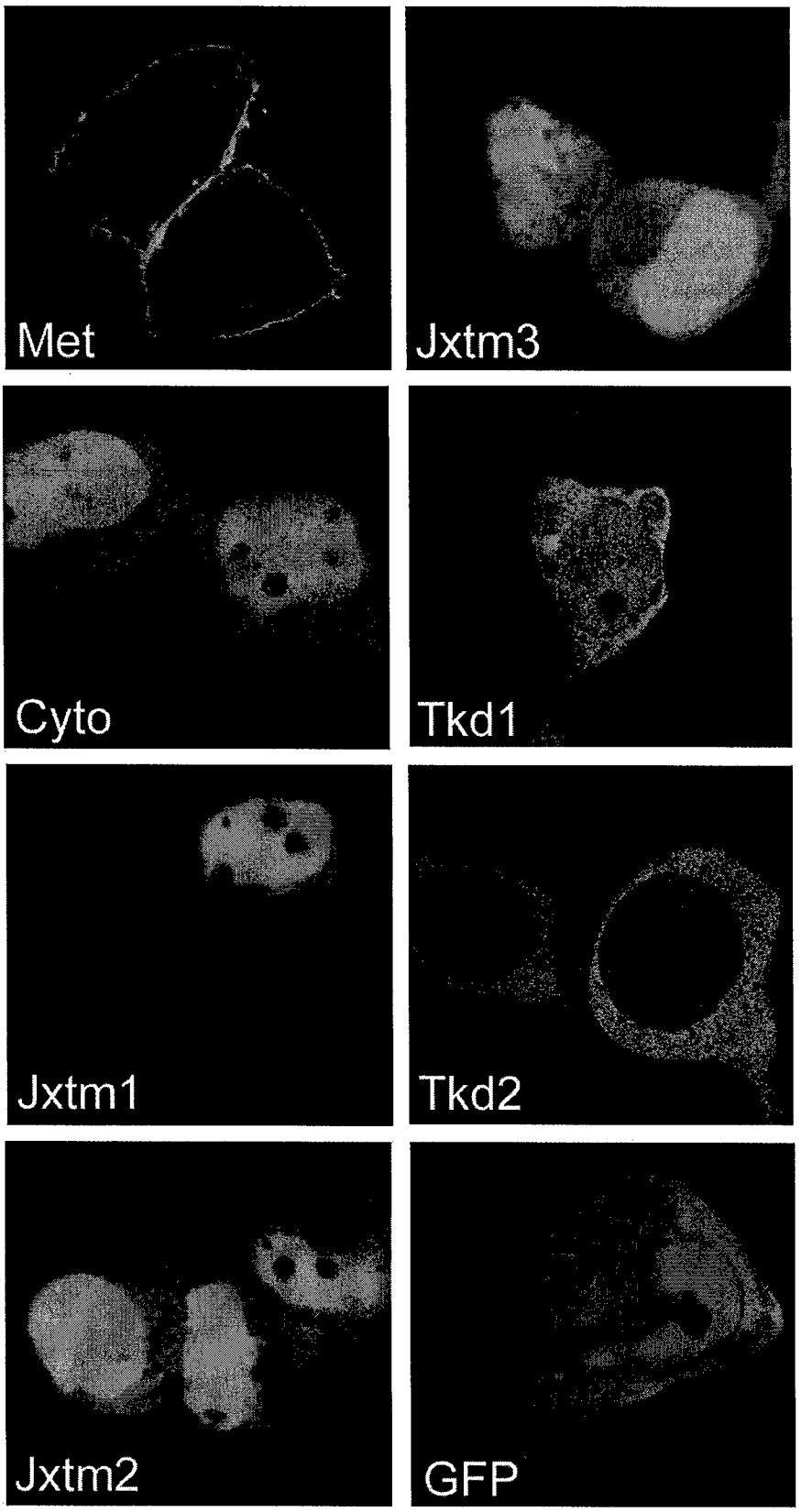

Figure 6